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Incorporation of Simian Virus 5 Fusion Protein into Murine Leukemia Virus Particles and Its Effect on the Co-incorporation of Retroviral Envelope Glycoproteins

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We describe the generation of murine leukemia virus (MLV) virus particles carrying the paramyxovirus fusion protein F from simian virus 5 (SV5-F). This glycoprotein was expressed in cells providing Moloney MLV (MoMLV) Gag and Pol proteins and a lacZ retroviral vector. SV5-F was correctly expressed, processed, and efficiently incorporated into retroviral particles. SV5-F-bearing retroviruses were not infectious although a weak binding to primate and rodent cells could be detected and SV5-F could mediate cell to cell fusion. We then co-expressed the SV5-F glycoprotein in retroviral particles with chimeric and wild-type MoMLV envelope glycoproteins. Our results show that F strongly inhibited infection via the retroviral envelopes although the mechanism of inhibition was different depending on the retroviral envelope used.

INTRODUCTION

Most retroviruses are thought to enter the target cells by direct fusion of the viral particle at the cell surface (McClure *et al.,* 1990). Fusion is mediated by the envelope glycoprotein of the retrovirus, which consists of two subunits: the surface subunit (SU), comprising the receptor binding domain, and the transmembrane subunit (TM), comprising the membrane anchor and the fusion peptide. Binding of the SU subunit to the cell surface receptor induces conformational changes in the envelope glycoprotein that lead to exposure of the fusion peptide, normally buried within the envelope trimer, and subsequent fusion of the viral and cell membranes. Like retroviruses, paramyxoviruses are thought to fuse at the cell surface (Lamb, 1993). In paramyxoviruses, however, the binding and fusion functions are separated into two different proteins, the attachment protein (generally hemagglutinin-neuraminidase (HN)) and the fusion protein F (Lamb, 1993). The exact mechanism by which paramyxoviral fusion proteins are activated remains unknown. Increasing evidence suggests that an interaction with the homotypic attachment protein (HN for simian virus 5 (SV5)) is required to trigger their fusogenicity (Deng *et al.,* 1995; Yao *et al.,* 1997). There are cases, however, such as simian virus 5, where the F protein can mediate fusion in the absence of HN (Horvath *et al.,* 1992).

We show here that the F glycoprotein from SV5-F could be efficiently incorporated into retroviral particles, yet the hybrid viral particles were not infectious. We then co-expressed SV5-F proteins with chimeric retroviral envelope glycoproteins that retarget virion binding. Viral titers obtained with viruses carrying these chimeras are generally low, probably due to a number of problems, including the poor fusogenicity of the chimeric glycoproteins (Cosset and Russell, 1996). We therefore proceeded to examine whether SV5-F could provide a helper fusion function when co-expressed with our Moloney murine leukemia virus (MoMLV)-based chimeric envelope glycoproteins and conversely whether the paramyxoviral attachment protein could be replaced by a wild-type or chimeric retroviral envelope glycoprotein in generating infectious viral particles.

RESULTS

Efficient incorporation of paramyxovirus envelope glycoproteins into retroviral particles. The simian virus type 5 fusion (SV5-F) envelope glycoprotein (Fig. 1) was transiently expressed in TELCeB6 cells (Cosset *et al.,* 1995b) that provide murine leukemia virus (MLV) retroviral core particles and a lacZ retroviral vector. SV5-F was found to be toxic to the cells and thus experiments were restricted to transient expression.

The fusion protein of paramyxoviruses is synthesized as a precursor, F0, subsequently cleaved by Golgi proteases in the host cell into two disulfide-linked subunits, F2 and F1. The simian virus 5 F protein forms trimers of F2–F1 heterodimers (Russell *et al.,* 1994). Western blots on the lysates of SV5-F-transfected cells were performed under nondenaturing conditions. Under these conditions the F2–F1 trimers remain associated and thus a high-molecular-weight band was obtained, migrating at about 205 kDa (Fig. 2A). The higher migrating bands observed probably correspond to SV5-F protein aggregates. A

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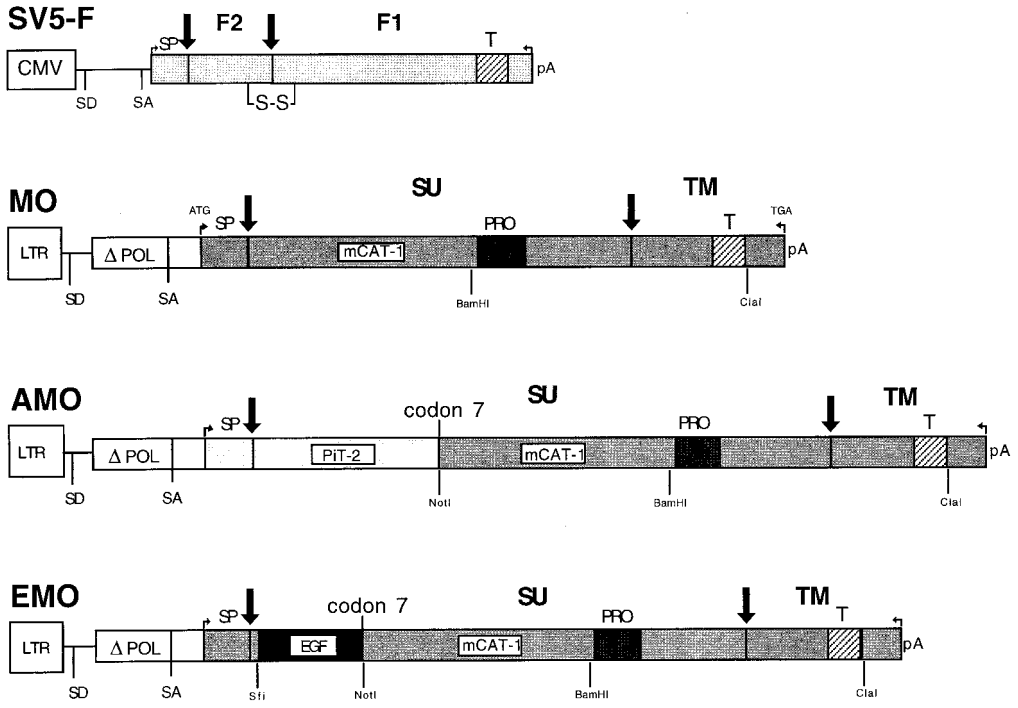

FIG. 1. Schematic diagrams of envelope expression constructs. The Simian virus 5 fusion protein SV5-F was expressed with the human cytomegalovirus (CMV) early promoter. Wild-type and chimeric retroviral envelope glycoproteins were expressed with the MoMLV long terminal repeat (LTR). The positions of some functional regions are indicated. Vertical arrows, protein cleavage sites; T, transmembrane domain; SP, env signal peptide. F2, N-terminal subunit of the SV5 fusion protein. F1, C-terminal subunit of the F protein comprising the transmembrane domain. MoMLV wild-type and chimeric envelopes: SU, surface protein; TM, transmembrane protein; PRO, polyproline hinge; mCAT-1, ecotropic MLV receptor binding domain; PiT-2, amphotropic MLV receptor binding domain; EGF, epidermal growth factor: Dark gray boxes, MoMLV-derived env sequences; light gray boxes, MLV-A-derived env sequences; white boxes, other MLV-derived sequences.

fainter band, around 83 kDa, was also obtained and probably corresponded to the SV5-F monomer (Fig. 2A).

To determine whether the SV5-F protein expressed was incorporated into the retroviral particles, immunoblot analysis was performed on pellets obtained by ultracentrifugation of supernatants of SV5-F-transfected cells. SV5-F trimers were detected in the viral particles pelleted from SV5-F-transfected TELCeB6 cells (Fig. 2A), but not in pellets from SV5-F-transfected TElac2 cells, which do not express Gag and Pol proteins (data not shown). This demonstrated that SV5-F proteins detected in pellets from TELCeB6 were associated with the retroviral particles. As a control, wild-type MLV envelope expression plasmid was transiently transfected in TELCeB6 cells. The MLV envelope glycoprotein is synthesized as a precursor cleaved in the Golgi into the SU and TM subunits. Two bands corresponding to the protein precursor and the SU were observed in immunoblots of cell lysates and in virion pellets (Fig. 2A). Relative to their respective levels of expression in cells, the efficiency of virion incorporation of SV5-F was slightly lower than that of the wild-type MLV envelope glycoprotein (Fig. 2A).

Fusion activity of the SV5-F protein. We wanted to determine whether the SV5-F protein expressed in TELCeB6 cells was fusogenic. On certain target cells, such as CV-1, SV5-F can induce the formation of syncytia independent of the presence of SV5-HN (Horvath *et al.,* 1992). Therefore, to verify the biological activity of SV5-F, cell to cell fusion assays were performed by co-cultivating CV-1 monkey or TE671 human indicator cells with TELCeB6 cells expressing the wild-type SV5-F gene. As expected, TELCeB6 cells that had been transfected with only the SV5-F expression construct could form syncytia with CV-1 indicator cells, although the syncytia were larger and more numerous when SV5-HN was co-expressed with SV5-F (Table 1). A mutant of SV5-F, G3A SV5-F (Horvath *et al.,* 1992), with enhanced fusogenicity was also expressed in TELCeB6 cells. The surface expression on TELCeB6 cells of both proteins was similar (data not shown). However, the number of syncytia obtained when cells expressing this mutant were co-cultivated with CV-1 cells was two times higher than when the wild-type SV5 F-transfected cells were used (Table 1).

As expected, TELCeB6 cells expressing the combination of both SV5-F and SV5-HN could easily fuse the TE671 indicator cells. However, in contrast to the observations with CV-1 indicator cells, no syncytia could be obtained when TELCeB6 cells expressing SV5-F alone, in the absence of SV5-HN, were co-cultivated with TE671 indicator cells (Table 1). Cells expressing the G3A SV5-F

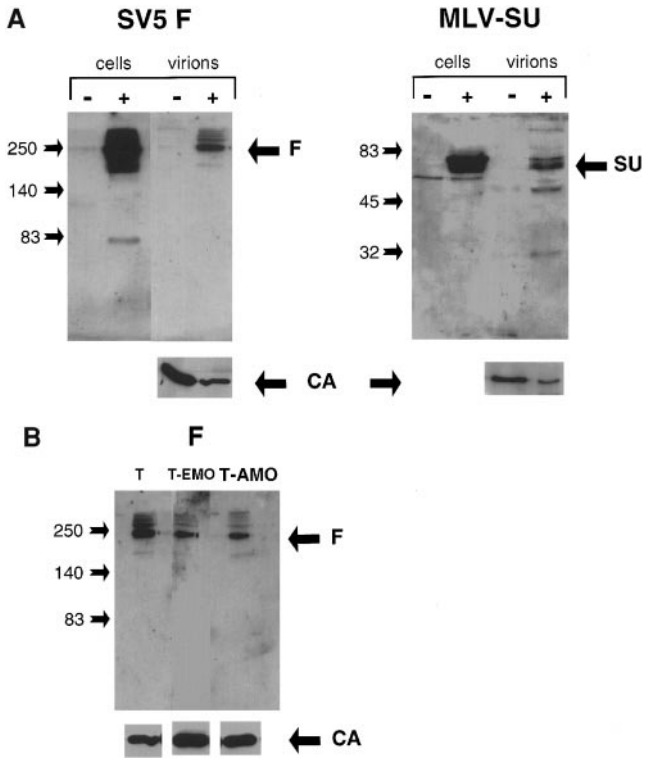

FIG. 2. Detection of envelope glycoproteins. (A) Immunoblots of lysates of TELCeB6 cells nontransfected (−) or transiently transfected (+) with the envelope glycoprotein indicated (depicted in Fig. 1) and of pellets of viral particles produced from these cells. Blots were stained with antibodies against the indicated proteins (see Materials and Methods). (B) Detection of SV5-F glycoproteins after co-expression with chimeric retroviral envelope glycoproteins. Immunoblots of pellets of viral particles produced from SV5-F transfected TELCeB6 (T), T-EMO, and T-AMO cells stained with anti-F antibody. For all of the immunoblots of virion pellets, the bottom panels (below 46 kDa) were stained with anti-p30 antibodies to detect the p30-CA capsid protein (CA).

fusogenic mutant were also unable to produce syncytia with TE671 cells (Table 1). Thus, although SV5-F was fusion competent, its ability to mediate fusion in the absence of SV5-HN depended on the nature of the target cells.

Co-expression of SV5-F and recombinant MoMLV-derived envelope glycoproteins into retroviral particles. To determine whether SV5-F could promote virus–cell fusion when co-expressed with heterologous attachment proteins, we then co-expressed the SV5-F proteins with chimeric retroviral envelope glycoproteins. Two retroviral chimeras were used: EMO and AMO. EMO and AMO (Fig. 1) are chimeric MoMLV-derived envelope glycoproteins designed to target the epidermal growth factor (EGF) receptor or the PiT-2 phosphate transporter, respectively (Cosset *et al.,* 1995a). Such chimeras can efficiently retarget the binding of retroviral particles onto which they are inserted to their respective targeted receptors, but cannot (EMO), or can barely (AMO), mediate a retargeted infection (Cosset *et al.,* 1995a).

The SV5-F glycoprotein expression construct was transiently transfected in T-AMO or T-EMO cells that stably express MLV core particles, a lacZ retroviral vector, and the AMO or the EMO chimeric retroviral envelope glycoproteins, respectively. Virions produced were analyzed by immunoblotting. The levels of incorporation of SV5-F in virions produced by SV5-F-transfected T-AMO or T-EMO cells were comparable to those found in virions produced by SV5-F-transfected TELCeB6 cells (Fig. 2B).

To further characterize the virions produced, supernatants of cells expressing EMO and/or SV5-F glycoproteins were incubated with A431 human cells, which overexpress EGF receptors. Binding of virions was then detected using an anti-EGF antibody, shown previously to reveal virion binding (Hatziioannou *et al.,* 1998). Virions generated with both EMO and SV5-F could bind A431 cells with a slightly lower efficiency than virions carrying EMO alone (Fig. 3A). This suggested that F co-expression did not significantly affect the ability of virion-associated EMO envelope glycoproteins to bind their target EGF receptors.

Additionally a weak binding of virions carrying SV5 F alone was revealed. This binding was also observed on a number of other cell lines tested, including human A431 cells and hamster Cerd9 cells (Fig. 3B). This binding could be due to the attachment of SV5 F to a yet unidentified receptor. Alternatively it could be due to the nonspecific attachment of F-carrying retroviral particles to the cell surface.

Co-expression of SV5-F with MLV envelope glycoproteins decreases the infectivity of the produced virions. Although retroviral particles carrying the SV5-F protein alone appeared capable of binding to the different cell lines tested, the infectious titers obtained with these viruses were <10 infectious units (iu)/ml on all the cell lines tested such as Cerd9, A431, TE671, and CV-1 cells. Subsequently the effect of SV5-F co-expression on the infectivity of retroviruses carrying the chimeric retroviral glycoproteins was studied.

TABLE 1

Cell to Cell Fusion Assays

Envelope expressed	Target cells	
	CV-1	TE671
No env	−	−
SV5-F	+	−
SV5-F + SV5-HN	++	+
SV5-HN	−	−
G3A SV5-F	++	−

Note. TELCeB6 cells expressing the different surface proteins indicated were co-cultivated with CV-1 or TE671 cells. Each plus sign represents 200 syncytia/cm^2.

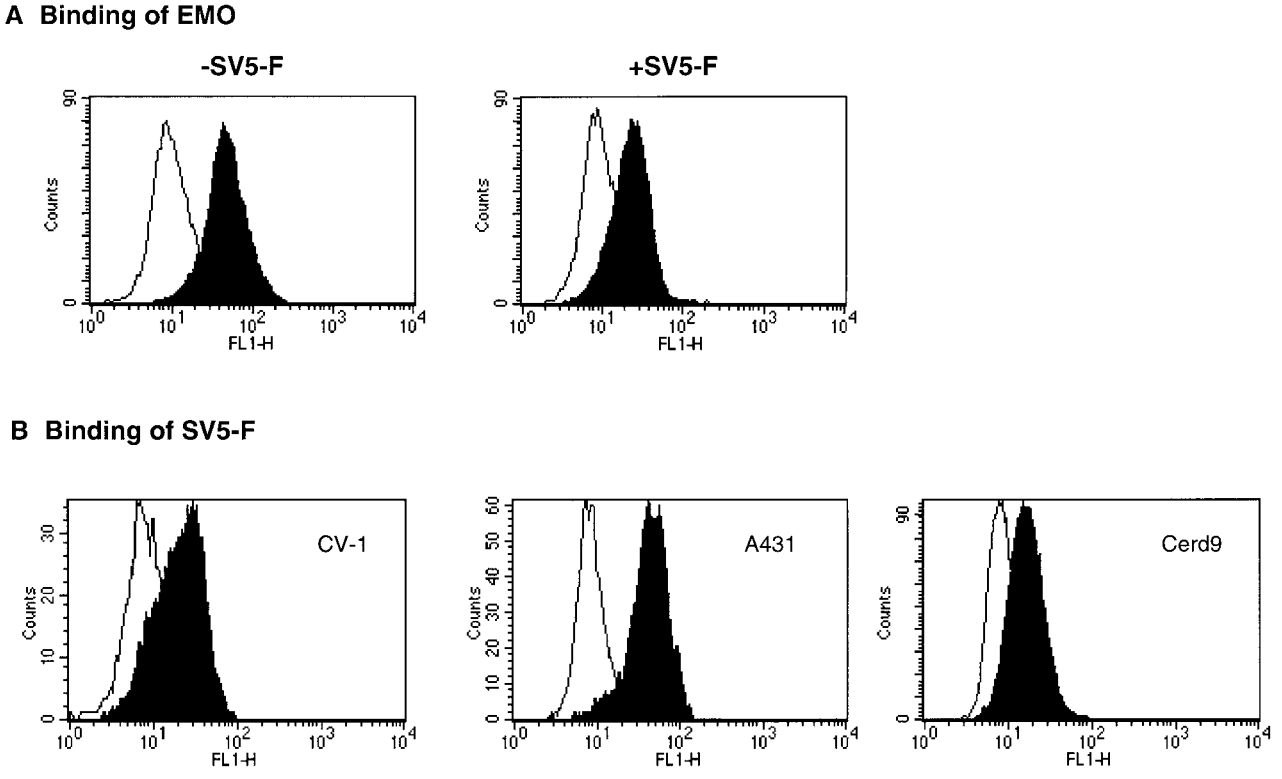

FIG. 3. Virion binding assays. (A) EGF receptor-mediated binding assays of virions coated with EMO or EMO + F glycoproteins on A431 cells. Cell-bound virions were stained with anti-EGF monoclonal antibody. (B) Binding assays of virions coated with SV5-F glycoproteins on Cerd9 (hamster), A431 (human), and CV-1 (monkey) cells. Cell-bound virions were stained with the 1A anti-F mouse monoclonal antibody.

As mentioned earlier, although chimeric envelope glycoproteins AMO and EMO efficiently retarget retrovirus binding, the infectivity of these chimeric viruses is poor (AMO viruses) to nonexistent (EMO viruses) (Cosset *et al.,* 1995a). In the case of AMO, the weak infectivity of the retargeted retroviruses is due mainly to the low fusogenicity of the chimeric retroviral glycoproteins (Cosset and Russell, 1996). Retroviruses carrying the chimeric AMO envelope glycoprotein can use the ecotropic virus receptor mCat-1 and/or the amphotropic receptor PiT-2 (Cosset *et al.,* 1995a). Retroviruses were generated with PiT-2-targeting AMO envelopes alone or in combination with the SV5-F glycoprotein and were used to infect PiT-2-expressing monkey CV-1 cells or human TE671 cells. Although retroviruses carrying AMO glycoproteins alone were infectious and had titers of 10^2 and 10^3 lacZ iu/ml on CV-1 and TE671 cells, respectively, viruses carrying both AMO and SV5-F glycoproteins could not infect these cells at all (Table 2). The same results were obtained when AMO was co-expressed with the highly fusogenic G3A SV5-F mutant glycoproteins (Table 2). The absence of infectivity on CV-1 cells was particularly surprising since both the wild-type and the mutant SV5-F proteins can mediate fusion of these cells, even in the absence of the paramyxoviral HN protein (Table 1).

Infection assays were also performed with retroviruses generated from producer cells co-expressing both SV5-F and EMO envelope glycoproteins. Binding of the EMO-carrying virions to the EGF receptors (EGFR) induces virus sequestration, contributing to the lack of infectivity of these viruses (Cosset and Russell, 1996). Hence EMO-bearing viruses are unable to infect EGFR-positive cells (Cosset *et al.,* 1995a). Retroviruses carrying both EMO and SV5-F envelope glycoproteins could not infect EGFR-positive A431 cells either, suggesting that

TABLE 2

Effect of SV5-F Expression on Infectivity of Viruses Produced from Cells Expressing Retroviral Envelope Glycoproteins

	Retroviral envelope expressed[a]				
	AMO		EMO		MO
Target cells[b]	CV-1	TE671	Cerd9	A431	Cerd9
−SV5-F	1×10^2	1×10^3	5×10^5	<1	1×10^7
+SV5-F	<1	<1	1×10^3	<1	1×10^2
+G3A SV5-F	<1	<1	nd	nd	nd

[a] SV5-F was transfected in cells expressing the indicated chimeric/wild-type retroviral envelope glycoprotein.

[b] Viruses were titrated on the target cells indicated. Titers are reported as lacZ iu/ml.

SV5-F could not rescue the infectivity of virions bound to EGF receptors (Table 2). Although they cannot infect human cells that express EGF receptors, retroviruses coated with EMO envelope glycoproteins can nevertheless easily infect EGF receptor-negative cells that express mCat-1 ecotropic receptors (Cosset *et al.,* 1995a), such as Cerd9 cells (Table 2). However, when infection assays were performed on Cerd9 cells, retroviruses that co-expressed both SV5-F and EMO glycoproteins had a strongly reduced infectivity compared to retroviruses generated with EMO envelopes alone by an order of magnitude of up to 2–3 logs (Table 2).

To further investigate this inhibitory effect of SV5-F on retroviral infection, we co-expressed SV5-F with the wild-type Moloney envelope glycoprotein. The effect of SV5-F co-expression on the infectivity of virions carrying wild-type MoMLV envelopes was even greater than that observed with the chimeric envelopes. Virions produced when both proteins were co-expressed had titers 5 logs lower than when the wild-type MoMLV glycoproteins were expressed alone. This suggested that SV5-F had a general fusion-inhibition activity when displayed on retroviral particles together with heterologous attachment glycoproteins.

Effect of SV5-F on incorporation of retroviral envelope glycoproteins. To determine why SV5-F expression had such an effect on the infectivity of virions when co-expressed with chimeric or wild-type retroviral glycoproteins the virion incorporation of the retroviral envelopes in the presence of SV5-F was analyzed. The presence of SV5-F in the viral particle was confirmed by immunoblots using the anti-F antibody (Fig. 4B).

Co-expression of SV5-F had no effect on the total amount of EMO chimeric envelopes incorporated into the virions produced; however, it did affect the processing of the TM subunit of the chimeric envelope (Fig. 4A). Similar results were obtained with the AMO envelopes (data not shown). Retroviral envelope glycoproteins are processed inside the virions by the viral protease, which removes a 16-amino-acid peptide from the C-terminus of the cytoplasmic tail, the R peptide. Removal of the R peptide has been shown to be essential for the fusogenicity of the envelope, and virions carrying nonprocessed envelopes are poorly infectious (Rein *et al.,* 1994).

The presence of unprocessed envelopes upon SV5-F expression could account in part for the lower infectious titers observed. The amount, however, of unprocessed envelope, compared to that of the processed envelope, is too low to explain the drastic decrease in infectious titer obtained. Thus SV5-F must have other effects acting at the level of viral entry, at a postbinding step.

On the other hand, co-expression of SV5-F had a different effect on the wild-type MoMLV envelope glycoproteins in that it decreased dramatically their incorporation in the produced virions (Fig. 4A). It thus seems that

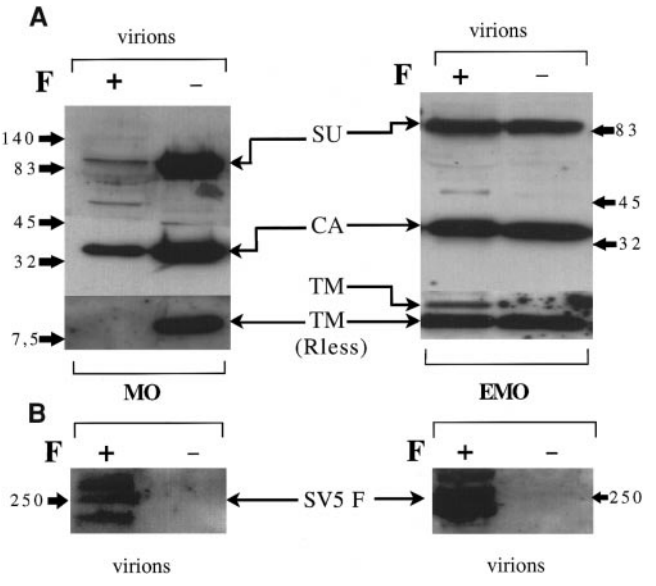

FIG. 4. Effect of SV5-F on virion incorporation of retroviral envelope glycoproteins. (A) Staining of retroviral glycoproteins. Immunoblots of pellets of viral particles from cells stably expressing wild-type MoMLV envelope glycoproteins (MO) or EMO chimeric envelope glycoproteins (EMO), transfected (+) or not (−) with the SV5-F expression plasmid. Staining was performed with antibodies against different retroviral proteins as indicated. (B) Staining of SV5-F proteins. Viral pellets were also run on a nondenaturing gel and stained with the anti-F monoclonal antibody.

the two envelopes compete for virion incorporation and SV5-F is preferentially incorporated (Fig. 4B), excluding the wild-type MoMLV envelopes. This "exclusion" accounts for the decreased titers obtained when both proteins are co-expressed. Similar results were obtained with other wild-type envelopes such as the 4070A and RD114 (data not shown).

DISCUSSION

Our results demonstrate that the fusion protein F from simian virus 5 can be efficiently incorporated into MLV particles, thus extending the list of nonretroviral glycoproteins incorporated into retroviral particles. The particles produced are not infectious despite a weak binding observed on the surface of primate and rodent cells. Furthermore, when SV5-F was coexpressed with retroviral envelope glycoproteins, it exhibited a strong inhibitory effect on the infectivity of the produced virions. This inhibition was due to different effects depending on the nature of the retroviral envelope glycoprotein. In the case of wild-type envelope glycoproteins, SV5-F competed with the retroviral envelope for virion incorporation and excluded the latter from being incorporated. The lower level of incorporation of wild-type envelope glycoproteins, in the presence of SV5-F, was not due to their lower expression levels in the cell (data not shown). Thus

SV5-F interferes with retroviral envelope incorporation during budding.

The exact mechanism of envelope incorporation in retroviral particles remains poorly defined. A specific interaction between the envelope glycoproteins and the Gag-Pol core has been suggested from studies on polarized cells (Lodge *et al.,* 1997; Weclewicz *et al.,* 1998). Studies on HIV-1 show that the envelope glycoprotein specifically interacts with the matrix protein of the Gag-Pol precursor via its cytoplasmic tail (Cosson, 1996) but no similar protein–protein interactions have been reported for MLV. MLV virions have been shown to efficiently incorporate envelope glycoproteins from other viruses such as vesicular stomatitis virus (Emi *et al.,* 1991), Rous sarcoma virus (Landau and Littman, 1992), Semliki Forest virus (Suomalainen and Garoff, 1994), human T-lymphotropic virus type 1 (Denesvre *et al.,* 1996), human foamy virus (Lindemann *et al.,* 1997), fowl plaque virus (Hatziioannou *et al.,* 1998), Ebola virus (Wool-Lewis and Bates, 1998), and HIV after truncation of the envelope glycoprotein cytoplasmic domain (Mammano *et al.,* 1997; Schnierle *et al.,* 1996). In these studies co-expression of the foreign glycoproteins with wild-type MLV was not examined. A more recent report demonstrated that the F protein from Sendai virus (paramyxovirus family) can also pseudotype retroviral particles and when co-expressed with a wild-type ecotropic retroviral envelope it reduced the infectivity of the virions produced (Spiegel *et al.,* 1998). Furthermore we have also obtained the same results when using the fusion protein from another member of the paramyxovirus family, the measles virus F protein (Hatziioannou and Cosset, unpublished data). Thus the inhibitory effect on the infectivity via retroviral envelope glycoproteins appears to be a common feature among F proteins from different paramyxoviruses.

The mechanism of exclusion by F is not clear, particularly since this effect is not observed when chimeric retroviral envelope glycoproteins are used. What we can conclude from our data is that the primary amino acid sequence of the cytoplasmic tail does not play a significant role during this process. Indeed the entire transmembrane subunit, comprising the cytoplasmic tail, of the chimeric retroviral glycoproteins AMO and EMO is identical to that of the wild-type MO envelope glycoprotein, yet the former are not excluded from the retroviral particles in the presence of SV5-F. The only difference between the chimeras and the wild-type envelope is that the former have an additional binding domain. It is likely that this addition influences the overall structure of the chimeras compared to the wild-type glycoproteins, rendering them more "resistant" to virion exclusion via F.

On the other hand, our data demonstrate that the SV5 fusion protein F is not fusion-active in the context of a retroviral particle. Although the fusion-triggering activity of paramyxovirus F proteins has not been clearly defined, two activation mechanisms have been proposed (Lamb, 1993). In the first model, the attachment protein HN brings F close to the target cell membrane where F can then mediate fusion either by recognition of an unidentified cell surface receptor or simply by contact with the target membrane. According to this model it should be feasible to replace the paramyxovirus HN by another attachment protein, such as a chimeric retroviral envelope glycoprotein. However, our data indicate that SV5-F cannot be functionally complemented by a retroviral attachment glycoprotein, at least in the context of a retroviral particle. Our data are therefore consistent with the second model of paramyxovirus F protein activation in which there is a physical interaction between HN and F, after HN has bound to its receptor, and this interaction activates F. A physical association between homotypic HN and F glycoproteins has been demonstrated for certain paramyxoviruses, suggesting that a highly specific interaction between the two proteins is required for F to be fusion active (Deng *et al.,* 1995). Therefore, in the absence of the corresponding HN to render F in its fusion active state, F may remain in a rigid conformation, behaving like a shield at the surface of the retroviral particle that prevents the close approximation of viral and cellular lipid membranes. However, other mechanisms for the inhibitory effect of SV5-F in the context of a retroviral envelope can be envisaged. For example, the F trimers could sterically hinder the conformational rearrangements of the retroviral envelope required for fusion to occur after receptor binding. Additionally they might interfere with the lateral mobility of the retroviral envelope proteins, thereby preventing them from clustering into a fusion pore. Alternatively, binding of F to the target cell surface might impede the mobility of the retroviral receptors, shown to be important for retroviral fusion. Finally our data do not exclude the unlikely possibility that SV5-F and retroviral glycoproteins may form nonfunctional mixed heterotrimers. In any case, this inhibitory effect appears to be specific for the paramyxoviral F protein since co-expression of the FPV (orthomyxovirus) HA protein with certain chimeric retroviral envelopes increased the infectivity of the virions produced compared to the infectivity of viruses carrying either protein alone (Hatziioannou *et al.,* 1998).

Although co-expression of both SV5-F and SV5-HN allowed the producer cells to fuse with target cells, the virions produced from these cells were not infectious (data not shown). Similar results were obtained with the surface proteins from measles virus (Hatziioannou and Cosset, unpublished data). In contrast Spiegel *et al.* (1998), showed that infection of an ecotropic packaging cell line with Sendai virus results in production of viruses exhibiting the Sendai virus tropism. This result suggests that both HN and F from Sendai virus are co-incorporated on the retroviral particle. However, it does not

exclude the possibility that other Sendai virus proteins might also be associated with the retroviral particles and contribute to their infectivity. Comparison between members of the paramyxovirus family has demonstrated in the past that different viruses have distinct requirements to mediate fusion (Bagai and Lamb, 1995). Expression of the paramyxovirus proteins in the context of retroviral particles might allow a better identification of the viral components required for the different paramyxoviruses to mediate virus to cell fusion.

MATERIALS AND METHODS

Cell lines. The TELCeB6 cell line (Cosset *et al.,* 1995b) was derived from TELac2 (Takeuchi *et al.,* 1994) after transfection and clonal selection of TE671 cells containing a plasmid expressing MoMLV gag and pol proteins. TELCeB6 cells produce noninfectious viral core particles, carrying an nlsLacZ reporter retroviral vector, whereas TELac2 cells express only the nlsLacZ reporter retroviral vector. T-EMO and T-AMO cells were derived from TELCeB6 cells that were stably transfected as previously described (Cosset *et al.,* 1995a) by plasmids expressing the EMO and the AMO chimeric envelope glycoproteins, respectively, and were further enriched by FACS cell sorting using anti-EGF (for EMO) or anti-gp70 (for AMO) antibodies.

TE671 (ATCC CRL8805) and A431 (ATCC CRL1555) human cells and CV-1 African green monkey kidney cells (ATCC CCL-70) were grown in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (Gibco BRL). Cerd9 (Kozak *et al.,* 1995) are derived from Chinese hamster ovary cells (ATCC CCL-61) and express the ecotropic MLV receptor (kind gift of D. Kabat). Cerd9 cells were grown in DMEM (Life Technologies) supplemented with 10% fetal bovine serum and with proline (Life Technologies).

Plasmids, transfection, and virus production. Plasmids encoding the EMO and AMO chimeric envelopes were described elsewhere (Cosset *et al.,* 1995a). Briefly, AMO consists of the Ram-1 binding polypeptide, provided by the first 208 amino acids of the MLV-A SU, fused at codon 7 of the MoMLV SU. EMO was constructed by inserting the sequence coding for EGF at the position corresponding to amino acid 7 of the MoMLV SU. The two binding domains carried by each chimeric envelope were separated from the wild-type receptor binding domain by a small linker containing three alanines. Both plasmids contain the phleomycin-selectable marker (Fig. 1). Wild-type and chimeric retroviral envelope glycoproteins were expressed using the MoMLV long terminal repeat promoter.

The SV5-F simian virus type 5 wild-type and G3A mutant (Horvath *et al.,* 1992) fusion glycoproteins (SV5-F and G3A SV5-F, kindly provided by R. Lamb) and the SV5-HN simian virus type 5 hemagglutinin-neuraminidase glycoprotein (SV5-HN, kindly provided by R. Lamb) were expressed using the hCMV-G (Yee *et al.,* 1994) expression vector (after excision of the VSV-G gene) under control of the human cytomegalovirus early promoter and rabbit β-globin intron sequences (Fig. 1).

Envelope glycoprotein expression plasmids were transfected by calcium phosphate precipitation into TELCeB6 cells as previously described (Cosset *et al.,* 1995a). Transfected cells were grown for about 48 h and virus-containing supernatants were collected after an overnight production from freshly confluent env-transfected TELCeB6 cells in regular medium.

Antibodies. Antibodies used included the following: anti-gp70 (Quality Biotech Inc., USA), a goat antiserum raised against the Rausher leukemia virus gp(SU)70, used diluted 1/2000 for Western blots; anti-CA (Quality Biotech Inc.), a goat antiserum raised against the Rausher leukemia virus p30 capsid protein (CA), used diluted 1/10,000 for Western blots; anti-TM antibody (a kind gift of Dr. A. Rein), which detects the transmembrane subunit of the MLV envelope glycoprotein, used diluted 1/1000 for Western blots; anti-EGF 3D3 (Boehringer Manheim), a mouse monoclonal antibody raised against EGF, used diluted 1/100 for FACS analysis; and anti-SV5-F (kindly provided by R. Lamb), a mouse monoclonal antibody 1A ascites fluid against SV5-F, used in FACS analysis, diluted 1/2000, and an anti-F_2 peptide rabbit serum used for Western blots, diluted 1/500.

Immunoblots. Virus producer cells were lysed in a 20 mM Tris–HCl buffer (pH 7.5) containing 1% Triton X-100, 0.05% SDS, 5 mg/ml sodium deoxycholate, 150 mM NaCl, and 1 mM PMSF. Lysates were incubated for 10 min at 4°C and were centrifuged for 10 min at 10,000 *g* to pellet the nuclei. Supernatants were then frozen at −70°C until further analysis. Virus samples were obtained by ultracentrifugation of viral supernatants (5 ml) in a SW41 Beckman rotor (30,000 rpm, 1 h, 4°C). Pellets were suspended in 50 μl of PBS (phosphate-buffered saline) and frozen at −70°C. Samples (30 μg for cell lysates or 20 μl for purified viruses) were mixed 5:1 (vol:vol) in a 375 mM Tris–HCl (pH 6.8) buffer containing 6% SDS, 30% β-mercaptoethanol, 10% glycerol, and 0.06% bromophenol blue, boiled for 3 min, and then run on 10% SDS acrylamide gels. For nonreducing gels the β-mercaptoethanol was omitted from the loading buffer, the samples were not boiled, the percentage SDS of the gel was reduced by half, and protein transfer onto nitrocellulose membranes was performed in the absence of methanol from the transfer buffer. After protein transfer onto nitrocellulose filters, immunostaining was performed in TBS (Tris base saline, pH 7.4) with 5% milk powder and 0.1% Tween. The blots were probed with the relevant antibody and developed using HRPO-conjugated Ig (immunoglobulins) raised against the species of each primary

antibody (Dako, UK) and using an enhanced chemiluminescence kit (Amersham Life Science).

Binding assays. Target cells were washed in PBS and detached by a 10-min incubation at 37°C with 0.02% versene in PBS. Cells were washed in PBA (PBS with 2% FCS and 0.1% sodium azide). A total of 5×10^5 cells were incubated with virus supernatant for 45 min at 37°C for F binding and at 4°C for EGF binding, in the presence of polybrene (5 μg/ml). Cells were then washed with PBA and were incubated with the anti-F 1A antibody or the anti-EGF antibody, respectively, for 45 min at 4°C. Cells were washed twice with PBA and incubated with anti-mouse Ig FITC-conjugated antibodies (Dako). Five minutes before the two final washes in PBA, cells were counterstained with 20 mg/ml propidium iodide. Fluorescence of living cells was analyzed with a fluorescence-activated cell sorter (FACSCalibur, Beckton Dickinson).

Cell–cell fusion assays. Transfected cells were detached, counted, and reseeded at the same concentration (3×10^5 cells/plate) in six-well plates. Fresh CV-1 or TE671 cells (1×10^6 cells per well) were then added to the transfected cells and were co-cultivated for 24 h. The co-culture was stained by adding the May-Grunwald and Giemsa solutions (Merck) according to the manufacturer's recommendations.

Infection assays. Target cells were seeded in 24 multiwell plates at a density of 5×10^4 cells per well. Viral supernatant dilutions containing 5 μg/ml polybrene were added and cells were incubated for 3–5 h at 37°C. Viral supernatant was then removed and cells were incubated in regular medium for 48 h. X-Gal staining and viral titer determination were performed as previously described (Cosset *et al.,* 1995a) as lacZ iu/ml.

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REFERENCES

Bagai, S., and Lamb, R. A. (1995). Quantitative measurement of paramyxovirus fusion: Differences in requirements of glycoproteins between simian virus 5 and human parainfluenza virus 3 or Newcastle disease virus. *J. Virol.* **69,** 6712–6719.

Cosset, F.-L., Morling, F. J., Takeuchi, Y., Weiss, R. A., Collins, M. K. L., and Russell, S. J. (1995a). Retroviral retargeting by envelopes expressing an N-terminal binding domain. *J. Virol.* **69,** 6314–6322.

Cosset, F.-L., and Russell, S. J. (1996). Targeting retrovirus entry. *Gene Ther.* **3,** 946–956.

Cosset, F.-L., Takeuchi, Y., Battini, J. L., Weiss, R. A., and Collins, M. K. L. (1995b). High titer packaging cells producing recombinant retroviruses resistant to human serum. *J. Virol.* **69,** 7430–7436.

Cosson, P. (1996). Direct interaction between the envelope and matrix proteins of HIV-1. *EMBO J.* **15,** 5783–5788.

Denesvre, C., Carrington, C., Corbin, A., Takeuchi, Y., Cosset, F.-L., Schulz, T., Sitbon, M., and Sonigo, P. (1996). TM domain swapping of murine leukemia virus and human T-cell leukemia virus envelopes confers different infectious abilities despite similar incorporation into virions. *J. Virol.* **70,** 4380–4386.

Deng, R., Wang, Z., Mirza, A. M., and Iorio, R. M. (1995). Localization of a domain on the paramyxovirus attachment protein required for the promotion of cellular fusion by its homologous fusion protein spike. *Virology* **209,** 457–469.

Emi, N., Friedmann, T., and Yee, J. K. (1991). Pseudotype formation of murine leukemia virus with the G protein of vesicular stomatitis virus. *J. Virol.* **65,** 1202–1207.

Hatziioannou, T., Valsesia-Wittmann, S., Russell, S., and Cosset, F.-L. (1998). Incorporation of fowl plague virus hemagglutinin into murine leukemia virus particles and analysis of the infectivity of the pseudotyped retroviruses. *J. Virol.* **72,** 5313–5317.

Horvath, C. M., Paterson, R. G., Shaughnessy, M. A., Wood, R., and Lamb, R. A. (1992). Biological activity of paramyxovirus fusion proteins: Factors influencing formation of syncytia. *J. Virol.* **66,** 4564–4569.

Kozak, S. L., Siess, D. C., Kavanaugh, M. P., Miller, A. D., and Kabat, D. (1995). The envelope glycoprotein of an amphotropic murine retrovirus binds specifically to the cellular receptor/phosphate transporter of susceptible species. *J. Virol.* **69,** 3433–3440.

Lamb, R. A. (1993). Paramyxovirus fusion: A hypothesis for changes. *Virology* **197,** 1–11.

Landau, N. R., and Littman, D. R. (1992). Packaging system for rapid production of murine leukemia virus vectors with variable tropism. *J. Virol.* **66,** 5110–5113.

Lindemann, D., Bock, M., Schweizer, M., and Rethwilm, A. (1997). Efficient pseudotyping of murine leukemia virus particles with chimeric human foamy virus envelope proteins. *J. Virol.* **71,** 4815–4820.

Lodge, R., Delamarre, L., Lalonde, J. P., Alvarado, J., Sanders, D. A., Dokhelar, M. C., Cohen, E. A., and Lemay, G. (1997). Two distinct oncornaviruses harbor an intracytoplasmic tyrosine-based basolateral targeting signal in their viral envelope glycoprotein. *J. Virol.* **71**(7), 5696–5702.

Mammano, F., Salvatori, F., Indraccolo, S., de Rossi, A., Chieco-Bianchi, L., and Göttlinger, H. G. (1997). Truncation of the human immunodeficiency virus type 1 envelope glycoprotein allows efficient pseudotyping of Moloney murine leukemia virus particles and gene transfer into $CD4^+$ cells. *J. Virol.* **71,** 3341–3345.

McClure, M. O., Sommerfelt, M. A., Marsh, M., and Weiss, R. A. (1990). The pH independence of mammalian retrovirus infection. *J. Gen. Virol.* **71,** 767–773.

Rein, A., Mirro, J., Haynes, J. G., Ernst, S. M., and Nagashima, K. (1994). Function of the cytoplasmic domain of a retroviral transmembrane protein: p15E–p2E cleavage activates the membrane fusion capability of the murine leukemia virus env protein. *J. Virol.* **68,** 1773–1781.

Russell, R., Paterson, R. G., and Lamb, R. A. (1994). Studies with cross-linking reagents on the oligomeric form of the paramyxovirus fusion protein. *Virology* **199,** 160–168.

Schnierle, B. S., Moritz, D., Jeschke, M., and Groner, B. (1996). Expression of chimeric envelope proteins in helper cell lines and integration into Moloney murine leukemia virus particles. *Gene Ther.* **3,** 334–342.

Spiegel, M., Bitzer, M., Schenk, A., Rossmann, H., Neubert, W. J., Seidler, U., Gregor, M., and Lauer, U. (1998). Pseudotype formation of Moloney murine leukemia virus with Sendai virus glycoprotein F. *J. Virol.* **72,** 5269–5302.

Suomalainen, M., and Garoff, H. (1994). Incorporation of homologous and heterologous proteins into the envelope of Moloney murine leukemia virus. *J. Virol.* **68,** 4879–4889.

Takeuchi, Y., Cosset, F. L., Lachmann, P. J., Okada, H., Weiss, R. A., and Collins, M. K. L. (1994). Type C retrovirus inactivation by human complement is determined by both the viral genome and producer cell. *J. Virol.* **68,** 8001–8007.

Weclewicz, K., Ekstrom, M., Kristensson, K., and Garoff, H. (1998). Specific interactions between retrovirus Env and Gag proteins in rat neurons. *J. Virol.* **72**(4), 2832–2845.

Wool-Lewis, R. J., and Bates, P. (1998). Characterization of Ebola virus entry using pseudotyped viruses: Identification of receptor-deficient cell lines. *J. Virol.* **72,** 3155–3160.

Yao, Q., Hu, X., and Compans, R. W. (1997). Association of the parainfluenza virus fusion and hemagglutinin-neuraminidase glycoproteins on cell surfaces. *J. Virol.* **71,** 650–656.

Yee, J. K., Friedmann, T., and Burns, J. C. (1994). Generation of high-titer pseudotyped retroviral vectors with very broad host range. *Methods Cell Biol.* **43,** 99–112.